

## Site-specific racemization in aging $\alpha$ A-crystallin

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Of all aspartyl residues in bovine  $\alpha$ A-crystallin, only Asp-151 exhibits pronounced racemization. Asp-151 is also one of the sites where peptide bond cleavage occurs in vivo aging  $\alpha$ A-crystallin. This aspartyl residue is followed by an alanyl residue and resides in a flexible carboxyl terminal extension of  $\alpha$ -crystallin. Both in vivo and in vitro racemization studies indicate that the pronounced and site-specific racemization of Asp-151 proceeds via formation of a succinimide intermediate. The in vivo racemization of aspartyl residues in  $\alpha$ A-crystallin is discussed with regard to the proposed tertiary structure of  $\alpha$ -crystallin.

Lens; Crystallin; Racemization; Molecular aging

### 1. INTRODUCTION

The vertebrate eye lens constitutes, due to the longevity of its proteins, a very suitable model for studying posttranslational modifications and aging of proteins [1–5]. The age-related modifications have most thoroughly been studied in  $\alpha$ -crystallin [4,6,7], which is a major structural protein in almost all vertebrate eye lenses. This protein is composed of 2 types of polypeptide chains,  $\alpha$ A and  $\alpha$ B, which are 173 and 175 residues in length, respectively, and show approximately 55% sequence homology [7,8].

We recently reported the deamidation and peptide bond cleavage at Asn-101 in bovine  $\alpha$ A-crystallin [9]. In this study, we showed that both the in vivo deamidation and cleavage occur via formation of a succinimide ring, which upon hydrolysis yields a mixture of normal and  $\beta$ -isomerized aspartyl residues and C-terminal asparagine and aspartic acid amide. These processes are accompanied by significant racemization.

Age-related racemization of L-aspartic acid, leading to the enantiomeric D-aspartic acid residue, has been observed in several long-lived proteins, like those in tooth enamel, dentine [10,11], proteins in the white matter of brain [12] and eye lens proteins [13–16]. However, in these studies the D/L ratio of total proteins or protein fractions was determined, with no correlation to individual aspartyl residues.

Increasing evidence has been provided that specific aspartyl and asparaginyl residues in peptides and proteins can undergo extremely rapid racemization via formation of a five-membered succinimide intermediate

[17], in contrast with the much slower racemization of aspartic acid, which proceeds by simple acid-base chemistry at the  $\alpha$ -carbon [18]. Therefore, it is of particular interest to examine the racemization of individual aspartyl residues. To gain insight in the in vivo extent of racemization of individual aspartyl residues in aging proteins, we determined the D/L ratio of all aspartic acid residues in bovine  $\alpha$ A-crystallin of different ages. Only a single aspartyl residue exhibits pronounced racemization. This aspartyl residue is also one of the sites, where peptide bond cleavage occurs in vivo aging  $\alpha$ A-crystallin.

### 2. EXPERIMENTAL

Bovine eye lenses, approximately 6 months and 5–8 years old, were obtained through the Central Animal Facilities of the University of Nijmegen, School of Medicine.

#### 2.1. Isolation and digestion of $\alpha$ A-crystallin

The lenses were dissected into cortex and nucleus, and subsequently homogenized in a buffered solution containing 50 mM Tris, 50 mM NaCl and 1 mM EDTA, pH 7.4. Water-soluble (WS) and urea-soluble (US) protein fractions were prepared by the extraction procedure as described by Bloemendal [18].  $\alpha$ A-Crystallin from calf lens cortex (6 months) and bovine lens nucleus (5–8 years) was isolated by gel permeation chromatography on Ultrogel AcA34 (Pharmacia LKB Biotechnology Inc.), followed by ion exchange chromatography on DEAE-cellulose (Whatman DE-52) or by carboxymethylcellulose (Whatman CM-52) as described previously [19].

The  $\alpha$ A-subunits were digested with trypsin and the resulting peptides were preparatively isolated by high voltage paper electrophoresis at pH 6.5 followed by descending chromatography [20]. After visualization with fluorescamine, peptides were identified by amino acid analysis. Two of the peptides, T13 and T18, could not be separated in this way, and were further analysed either by high voltage re-electrophoresis at pH 3.5 or by thermolytic digestion at 50°C in 0.1 M  $\text{NH}_4\text{HCO}_3$ , pH 8.9 using a peptide concentration of 0.5 nmol/ $\mu$ l. The resulting thermolytic peptides were separated by high voltage electrophoresis at pH 6.5 [20].

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Table I

D/L ratio of aspartyl residues of native  $\alpha$ A-crystallin, isolated from water-soluble fractions of calf lens cortex (WS cortex, 6 months) and bovine lens nucleus (WS nucleus, 5 years), and from the urea-soluble fraction of bovine lens nucleus (US nucleus, 5–8 years). Values are the mean of triplicate measurements

Tryptic peptides containing aspartyl residues as indicated		WS Cortex (6 months)	WS Nucleus (5 years)	US Nucleus (5–8 years)
T1	: ac-Met-Asp <sup>2</sup> -Ile-Ala-Gln-His-Pro-Trp-Phe-Lys	0.014	0.027	n.d.
T4	: Leu-Phe-Asp <sup>24</sup> -Gln-Phe-Phe-Gly-Glu-Gly-Leu-Phe-Glu-Tyr- Asp <sup>35</sup> -Leu-Leu-Pro-Phe-Leu-Ser-Ser-Thr-Ile-Ser-Pro-Tyr- Tyr-Arg	n.d.	0.031	n.d.
T6	: Thr-Val-Leu-Asp <sup>58</sup> -Ser-Gly-Ile-Ser-Glu-Val-Arg	0.012	0.033	0.080
T7–8	: Ser-Asp <sup>67</sup> -Arg-Asp <sup>69</sup> -Lys	0.017	0.021	0.042
T9	: Phe-Val-Ile-Phe-Leu-Asp <sup>76</sup> -Val-Lys	n.d.	0.018	n.d.
T10	: His-Phe-Ser-Pro-Glu-Asp <sup>84</sup> -Leu-Thr-Val-Lys	0.010	0.019	n.d.
T11	: Val-Gln-Glu-Asp <sup>92</sup> -Phe-Val-Glu-Ile-His-Gly-Lys	0.012	0.017	0.025
T13	: Gln-Asp <sup>105</sup> -Asp <sup>106</sup> -His-Gly-Tyr-Ile-Ser-Arg	0.021	0.046	0.073
T17a	: Leu-Pro-Ser-Asn-Val-Asp <sup>125</sup> -Gln-Ser-Ala-Leu-Ser-Cys	0.012	0.029	0.047
T17b/T10x <sup>1</sup>	: Ser-Leu-Ser-Ala-Asp <sup>136</sup> -Gly-Met-Leu-Thr-Phe-Ser-Gly-Pro-Lys / ac-Met-Asp <sup>2</sup> -Ile-Ala-Gln-His-Pro-Trp-Phe-Lys	n.d.	0.021	0.031
T17b/T10 <sup>1</sup>	: Ser-Leu-Ser-Ala-Asp <sup>136</sup> -Gly-Met-Leu-Thr-Phe-Ser-Gly-Pro-Lys /His-Phe-Ser-Pro-Glu-Asp <sup>84</sup> -Leu-Thr-Val-Lys	0.013	n.d.	0.032
T18	: Ile-Pro-Ser-Gly-Val-Asp <sup>151</sup> -Ala-Gly-His-Ser-Glu-Arg	<u>0.040</u>	<u>0.327</u>	<u>0.493</u>
T18a <sup>2</sup>	: Ile-Pro-Ser-Gly-Val-Asp <sup>151</sup>	n.d.	0.027	n.d.

<sup>1</sup> T17b, both non-oxidized and oxidized form, could not be recovered as a single peptide, but was contaminated with T10x and T10, respectively.

<sup>2</sup> In vivo cleavage product:  $\alpha$ A-(1–151)-crystallin.

n.d.: not determined.

## 2.2. In vitro racemization of $\alpha$ A-crystallin

Purified  $\alpha$ A-crystallin from the water-soluble fraction of calf lens cortex (6 months) was incubated under vacuum in a concentration of 10 nmol/150  $\mu$ l in 0.1 M sodium phosphate buffer, pH 7.4 at 70°C [17]. Incubations were terminated at different time intervals by short dialysis and subsequent lyophilization. D/L-Asp ratios were determined after isolation of Asp-containing peptides as described.

## 2.3. Analysis of aspartic acid enantiomers

The D/L-Asp ratios of peptides were determined by high performance liquid chromatography after acid hydrolysis for 6 h at 110°C and after conversion of D/L enantiomers into diastereomers as described [21]. No corrections for background racemization were made, because the racemization rate of individual aspartyl residues during acid hydrolysis strongly depends on their local environment [22].

## 3. RESULTS AND DISCUSSION

D/L aspartic acid ratios of all individual aspartyl residues of water-soluble and urea-soluble  $\alpha$ A-crystallin were determined (Table I). Among the 14 aspartyl residues of the  $\alpha$ A-subunit of  $\alpha$ -crystallin, only a single one, at position 151, exhibits pronounced racemization. As illustrated in Fig. 1, the enantiomeric

ratio of Asp-151 increases from 0.040 in water-soluble  $\alpha$ -crystallin from calf lens cortex to 0.327 and even to 0.493 in water-soluble and urea-soluble  $\alpha$ A-crystallin from bovine lens nucleus, respectively. Since racemization is an age-dependent process, the increase in racemization from water-soluble to urea-soluble  $\alpha$ -crystallin provides evidence that  $\alpha$ -crystallin becomes progressively less water-soluble with increasing age, as previously indicated by others [23]. The D/L ratios of all other aspartyl residues range from 0.010 to 0.021 in young water-soluble  $\alpha$ A-crystallin and increases to 0.025 to 0.080 in urea-soluble  $\alpha$ A-crystallin of old bovine nucleus. This slow increase in racemization can be attributed to simple acid-base chemistry at the  $\alpha$ -carbon, whereas the pronounced and site-specific racemization of Asp-151 will be the result of formation of a succinimide intermediate. This is strongly confirmed by in vitro racemization studies. Table II shows that only Asp-151 racemizes upon incubation of  $\alpha$ A-crystallin, under conditions that favor succinimide formation. The D/L ratios of other aspartyl residues hardly increase upon in vitro incubation.

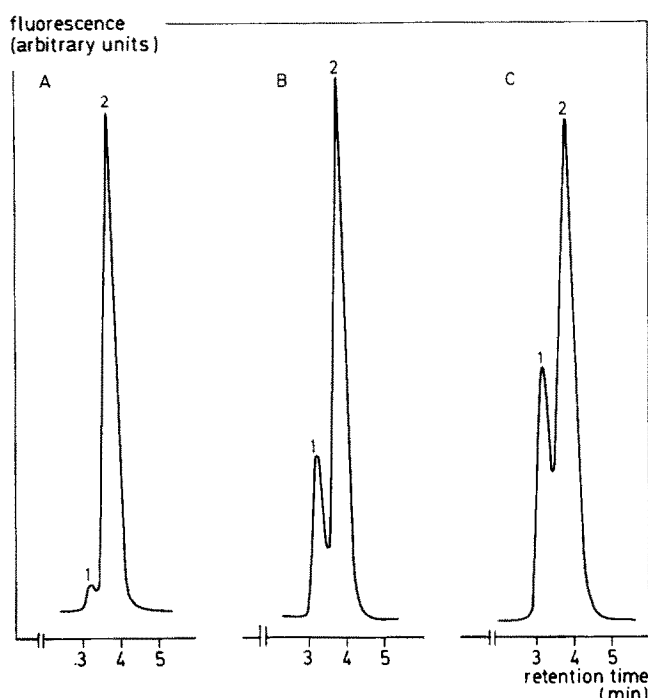


Fig. 1. Diastereomeric HPLC separation of T18 hydrolysate, isolated from water-soluble fractions of calf lens cortex (A) and bovine lens nucleus, 5 years (B), and from urea-soluble fraction of bovine lens nucleus, 5-8 years (C). Separation was performed on a reversed-phase HPLC column (C18, Chrompack). Diastereomeric derivatives were eluted isocratically with 50 mM sodium acetate pH 5.7, 3.2% methanol. 1 = diastereomeric derivative of D-aspartic acid. 2 = diastereomeric derivative of L-aspartic acid.

Studies with model peptides have shown that succinimide formation is highly dependent on the local conformation and the neighboring residues.  $\alpha$ A-Crystallin contains Asp-Ser and Asp-Gly sequences (T6 and T17b peptides) that have the chemical susceptibility of imide ring formation [17,24]. However, in  $\alpha$ A-crystallin, these aspartyl residues have low D/L ratios. Our results thus show that it is difficult to predict the rate of succinimide formation from sequence data alone. The local conformation of aspartyl or

Table II

D/L ratio of aspartyl residues of  $\alpha$ A-crystallin, after in vitro incubation in 0.1 M sodium phosphate buffer, pH 7.4 at 70°C. The D/L ratio of T18, containing the in vivo fast racemizing Asp-151, and of several control peptides was determined. Values are the mean of two independent incubations, whereas D/L ratios were measured in triplicate

Tryptic peptides	Incubation time (days)	D/L-Asp ratio
T18	0	0.040
	6	0.079
	19	0.158
T7-8	19	0.029
T10	19	0.040
T11	19	0.044

asparaginy residues in peptides and proteins may be of much more importance in determining the rate of succinimide formation [22,25].

Because of the strong conformational constraints, succinimide-linked reactions occur most prevalently in partially denaturated or conformationally flexible regions of proteins [22]. The extreme and site-specific racemization of Asp-151 in  $\alpha$ A-crystallin suggests that this region is located in a conformationally flexible region of the protein. This is indeed supported by earlier work, which provided evidence that Asp-151 resides in the flexible surface-exposed carboxyl-terminal arm of  $\alpha$ -crystallin [19,26,27]. All other aspartyl residues reside in specific  $\alpha$ -crystallin domain structures and may be involved in formation and stabilization of the tertiary structure of globular  $\alpha$ -crystallin. Asp-2, Asp-35, Asp-69 and Asp-105 are considered to be of potential structural importance, as they are extremely well conserved among  $\alpha$ -crystallins of different animals [27]. These  $\alpha$ -crystallin domain structures may be extremely stable and therefore do not permit succinimide formation at aspartyl residues.

Interestingly, Asp-151 is also one of the sites, in addition to Asn-101, where peptide bond cleavage occurs in aging  $\alpha$ A-crystallin [28]. It is therefore tempting to speculate that the cleavage between Asp-151 and Ala-152 might proceed nonenzymatically via intramolecular ring formation, which has already been demonstrated in vivo at Asn-101 in  $\alpha$ A-crystallin [9] and in vitro at a specific aspartyl residue of calmodulin [29]. The peptide bond cleavage at Asn-101 in  $\alpha$ A-crystallin was shown to proceed via formation of a C-terminal succinimide ring and was accompanied by enhanced racemization. Whether the in vivo cleavage at Asp-151 also occurs by intramolecular cyclization, via a succinic anhydride intermediate, is still not clear. Although the D/L ratio of Asp-151 in the in vivo truncated  $\alpha$ A-(1-151)-chain is low, an enzymatic process is not very plausible, because no enzyme is known to bring about such cleavage. Further experiments are needed to elucidate the cleavage mechanism at this aspartyl residue.

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